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CBP in Wnt-signaling Dependent Mammary Carcinogenesis

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Wnt signaling is mediated by a multi-component cascade that relays the signal from cell membrane to nuclear TCF- β -catenin transcriptional complex. Genetic study has implicated CREB-Binding Protein (CBP) as a negative regulator of Wnt signaling, suggesting that CBP could modulate the wnt-induced carcinogenesis. In contrast to our original hypothesis that CBP-mediated acetylation negatively regulates TCF-dependent transcription, we have previously found that CBP potentiates TCF- β -catenin transcriptional activity by physically interacting with β -catenin. We then hypothesize that in the absence of β -catenin, CBP facilitates the function of TCF as a transcriptional repressor in the context of chromatin. Upon binding to β -catenin, CBP is converted to a classical transcriptional co-activator that activates wnt-dependent downstream target genes. To investigate this possibility, we have created analyzed chromatinized reporters based on chromosomal reporter or by replication-competent reporter plasmid. Preliminary study on these reporters indicate that TCF binding sites confer transcriptional repression activity. We are now investigating whether CBP and TCF members modulates this repression activity. On the TCF4 acetylation front, by analyzing TCF acetylation pattern, we obtained evidence that there are more than one acetylation sites present in TCF4. We are currently mapping the novel acetylation sites and analyze their potential functions.

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Progress Report:

Title: Functional analysis of the transcriptional co-activator CBP in Wnt-signaling dependent mammary carcinogenesis

Introduction

The long-term objective of this proposal is to analyze the role of the transcription co-activator CBP in Wnt-signaling dependent carcinogenesis. In mouse model, wnt over-expression in mammary gland results in the development of breast carcinoma. Genetic study in *Drosophila* has implicated CBP as a negative regulator of TCF transcription factor, the major downstream effector of wnt signaling. As heterozygous mutant CBP mice develop mammary gland hyperplasia, these observations suggest that CBP might play a negative role in regulating wnt-dependent mammary gland carcinogenesis. Molecularly, TCF can function both as a transcription repressor and activator. In the absence of wnt, TCF actively suppresses the wnt-responsive genes. However, wnt activation leads to the formation of β -catenin –TCF complex, which functions as a transcriptional activator on wnt-responsive genes. In our proposal, we hypothesized that CBP negatively regulates wnt-signaling by either dominantly suppressing TCF- β -catenin -dependent transcriptional activation or by promoting the transcriptional repressor function of TCF. Some of these issues were addressed in the first 24 months of the award period.

Body:

The main research objectives of the last twelve months are two folds.

First, to set up an assay to assess the role of TCF4 and CBP-mediated acetylation in transcriptional repression.

Second, to begin to assess the function of TCF4 acetylation.

We have made progresses at both fronts.

1. To investigate the potential role of CBP-TCF mediated transcriptional repression.

Genetic results from both *Drosophila* and *Xenopus* have suggested that TCF might actively repress wnt-target gene expression [1]. Furthermore, this repression appears to involve CBP [1]. We have proposed to reconstitute this transcriptional repression system in tissue culture system to further dissect out the molecular details of this repression. Despite the repeated effort, however, a TCF-reporter assay (TOP-flash, OT) in a transient transfection setting failed to reveal any TCF dependent transcriptional repression (This part of study was reported in last year's report). As transcriptional repression often involves specific repressive chromatin configuration (reviewed in [2]), we reasoned that it might be necessary to utilize chromatinized reporter to establish the transcriptional repression assay, as it is not likely that reporter plasmid in a transient transfection setting will be efficiently assembled into chromatin. To this end, we have taken three independent approaches to establish chromatinized reporter system to assess TCF, CBP and β -catenin function.

- a. To generate cell lines with TCF reporter stably integrated to genome. The stably integrated reporter genes are likely to be organized into proper chromatin structure and therefore confer TCF-dependent repression. To achieve this, we have transfected TCF response element driven luciferase (OT) or mutant reporter (OF) with a selectable marker: puromycin resistant gene, and selected for the puromycin-resistant stable clones. We analyzed more than 10 independent clones for cells stably transfected with either OT or OF reporter. Although, as expected, there are variations in the transcriptional levels among clones, in general, OT stable lines express much lower basal transcriptional activity when compared with OF. We have found that more than 50% of the OT expressing stable lines display considerable lower basal transcriptional activity. The analyses of a few stable lines are shown in Figure 1, wherein OT lines show lower basal transcriptional activity. This observation is consistent with the idea that TCF binding sites can mediate transcriptional repression in the absence of β -catenin. Note that OT, but not OF, can be activated by the introduction of TCF4 and β -catenin, demonstrating that these clones have functional TCF-reporter gene integrated.

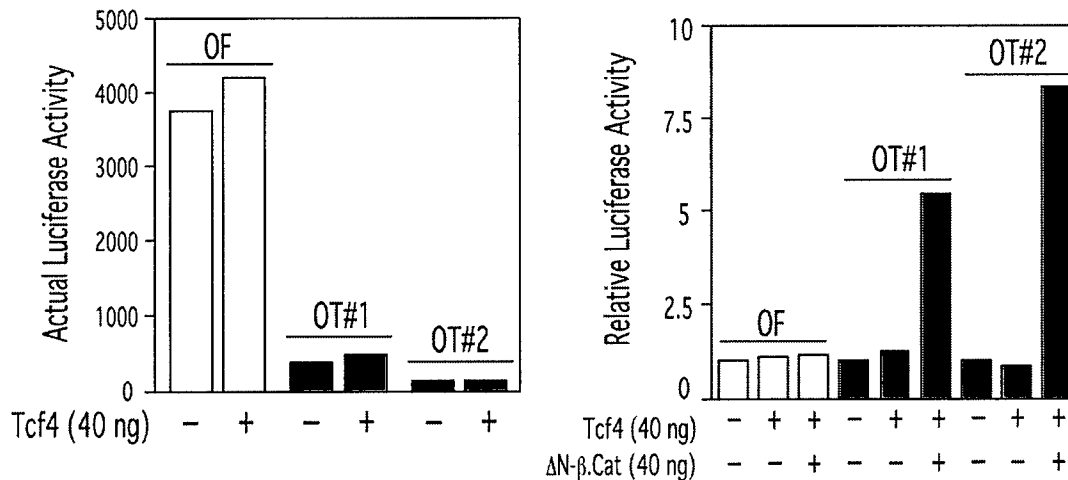


Figure1. Transcription repression by Tcf and activation by Δ N- β .Catenin in H1299 stably expressing TCF reporter gene. H1299 cells with pGL3-OF/OT luciferase reporter stable expression were transiently transfected with Tcf4 alone or plus activated form of β .Catenin (Δ N). Forty-eight hours post-transfection, cell extracts were subjected to luciferase assay. Luciferase activity is shown in fold induction and pGL3-OF stable line without transfection of Tcf4 or/and Δ N- β -catenin is arbitrarily designated as 1.

- b. To assess a TCF-reporter plasmid which can actively replicate. As plasmid DNA that undergoes replication is known to be assembled into nucleosome, we cloned the TCF response element into an episomal vector that contains Epstein-Barr virus replication origin and encodes the nuclear antigen EBNA-1 (PREP-4 luciferase) [3]. As shown in Figure 2, wild type TCF response element (OT) driven luciferase cloned into this vector (pPREP4) does display much lower transcriptional activity when compared with that of the mutated reporter (OF). This result provides another line of evidence that TCF does repress transcription in the absence of active wnt signaling. Surprisingly, however, additional expression of TCF4 by transfection did not confer further transcriptional repression. There are at least two testable explanations for this observation. First, other TCF family member, such as TCF1 or LEF1 but not TCF4 is responsible for the repression. This will be addressed by assessing whether transfection of TCF1 or LEF1 expression plasmids can lead to a further reduction of the reporter activity. Second, the endogenous level of TCF4 is sufficient to mediate repression. This possibility can be examined by over-expression of a dominant negative TCF4 and determine if this mutant can lead to the de-repression of this reporter.

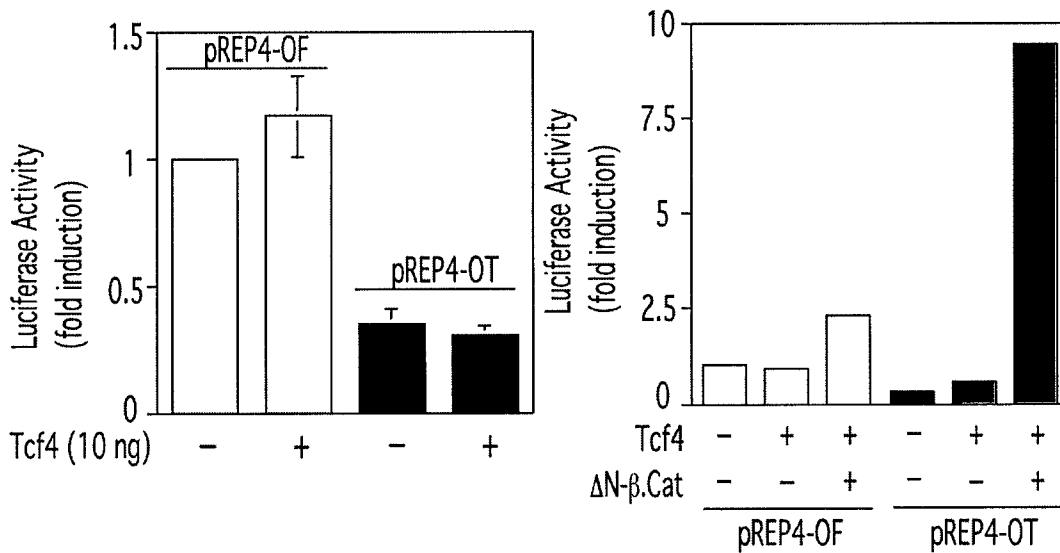


Figure 2. TCF binding sites confers transcriptional repression in the context of pREP4. Tcf4 responsive elements contained three copies of the optimal Tcf motif CCTTTGATC (designed as OT), or three copies of the mutant motif CCTTTGGCC (designed as OF) were cloned into pREP4-Luciferase episomal vector that contains Epstein-Barr virus replication origin and encodes nuclear antigen EBNA-1. These plasmids can be incorporated into nucleosomes upon its replication. H1299 cells were transiently transfected with pREP4-OF/OT luciferase reporter with or without Tcf4 or β -catenin as indicated. Forty-eight hours post-transfection, cell extracts were subjected to luciferase assay. Luciferase activity is shown in fold induction by arbitrarily designating transfection with pGL3-OF luciferase reporter alone as 1. Note that PREP4-OT has much lower basal transcriptional activity than PREP -OF, suggesting an active repression mediated by TCF binding element (Left Panel). Both PREP4-OT and PREP4-OF responds to TCF4 and Δ N- β -Catenin normally (Right Panel).

- c. To investigate the TCF transcriptional response in *Xenopus* oocyte system. It has been well-established that plasmid injected in *Xenopus* oocytes are properly chromatinized[4]. This system provides an ideal assay system to investigate the repression-activation transition on TCF-mediated transcription. We have already cloned TCF, β -catenin and OT and OF reporter into vectors that are suitable for *Xenopus* oocyte assay. We will perform these experiments with the help from Dr. Jiemen Wong, whose laboratory is one of the first to use this system to document the transcriptional repression and activation of nuclear hormone receptor [5]. With this powerful system, we expect to obtain important insight

regarding the role of TCF and CBP-mediated acetylation in transcriptional repression.

With these assay systems in hand, we are in the process to assess whether TCF4 or its related family member, such as LEF1 and TCF1 might repress TCF reporter in the chromatinized configuration. Furthermore, we will also utilize dominant negative mutant for each of these TCF members [6] to further assess the role of individual TCF family member. The goal is assess whether over-expression of these mutants can relieve the transcriptional repression. A positive result here will provide further supporting evidence that specific members of TCF are responsible for the active repression of wnt target genes at the basal state. Lastly, we will address whether CBP-mediated acetylation is critical for this repression. This can be achieved by two ways. First, we will determine if the TCF4 acetylation mutant (KA) can still repress transcription (but see the evidence of the existence of additional acetyltable lysine residues in TCF4 in the next section). Second, we will test whether CBP deficient in acetyltransferase activity can still promote TCF dependent repression. The *Xenopus* oocyte system might be particularly suitable to address this question, as we have used this assay to demonstrate that an acetyltransferase-deficient p300 failed to modulate the nuclear hormone receptor transcriptional activity (Lai, C.-H, Huang, Z.Q.*, Dougherty, R*.. Wong, J.M., and Yao, T.-P. Submitted). We expect this system to be similarly competent to address the importance of the acetyltransferase activity of CBP.

2. To assess the function of TCF4 acetylation.

The evolutionarily conserved Lysine 22 in TCF4 is believed to be acetylated by CBP [1]. To begin to investigate the function of this specific acetylation, we have mutated this lysine to alanine (KA(22)). However, as shown in Figure 3, this mutation has no apparent effect on the transcriptional proerty of TCF4. We thus suspect that there might be more than one lysine residues subject to modification by CBP. Therefore, mutation on one lysine might not be sufficient to create a dominant phenotype. A similar conclusion was obtained by our previous study on p53 acetylation ([7], Ito, et. al., submitted).

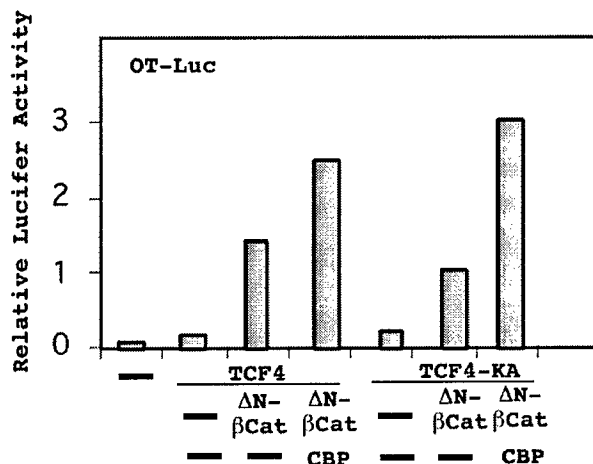


Figure 3. TCF4-KA (22) mutant retains wild type TCF4 transcriptional activity.

Expression plasmid for wild type TCF4 or acetylation deficient KA(22) mutant are co-transfected with reporter plasmid in the presence or absence of β -catenin and CBP. Note that TCF4-KA has similar transcriptional activity under the condition tested.

To further explore the possibility that there might be additional lysine residues, other than lysine 22, can be acetylated by CBP, we generated recombinant TCF4-KA(22) protein and test whether it is still a substrate for CBP. As shown in Figure 4, TCF4-KA(22) is efficiently acetylated by CBP in vitro. This result indicates that there are additional lysine residues that can be acetylated by CBP. To begin to map the additional acetylated lysine residues, we evaluate recombinant fragments of TCF4 in CBP-mediated acetylation assay. To our surprise, C-terminal TCF4, which does not contain K22, is strongly acetylated. This result indicates that novel acetylation site(s) present at the C-terminus of TCF-4. In collaboration with Dr. Eppella's lab at NIH, with whom we have mapped the MDM2 acetylation sites, we will begin to identify the acetylated lysine residues in TCF4. Mapping these sites will be essential to dissect the function of TCF4 acetylation.



Coomassie Blue Stain Autoradiography

Figure 4. CBP-mediated TCF4 acetylation in vitro. The substrates, purified recombinant GST fusion Tcf4 N'-terminal fragment (1-300), C'-terminal fragment (301-596), full length wild-type Tcf4, or full length Tcf4.K22A mutant from *E. coli*, were incubated with baculoviral recombinant CBP in HAT reaction buffer with [14 C]-acetyl-CoA. The reaction products were subjected into SDS-PAGE and then followed with Coomassie brilliant blue staining (left panel) and autoradiograph (right panel). Note that TCF4KA (22) is efficiently acetylated.

Key Research Accomplishment:

1. Establish the chromatinized wnt/TCF responsive reporter system for the study of the roles of various TCF family member and CBP-mediated acetylation in active repression of TCF/wnt target genes.
2. Establish that the acetylation of lysine 22, the putative major acetylation site in TCF4, is not necessary for its function as a transcription activator.
3. Establish that lysine 22 of TCF4 is not the only lysine residues that can be acetylated by CBP. This result suggests that, similar to most of known acetylated proteins, there will be additional lysine residues can be modified by acetylation.

Reportable Outcome:

Lai, C.-H, and Yao, T.-P. Transcriptional co-activator CBP interacts with β -catenin to control TCF transcriptional activity. **(In Preparation)**.

Conclusion:

Based on our study during the first year of this grant period, we proposed that TCF likely plays dual role in the regulation of wnt target genes. We hypothesized that in the absence of wnt, in collaboration with CBP, TCF represses wnt target gene expression. In the presence of wnt, which leads to nuclear accumulation of β -catenin, the transcriptional complex TCF-CBP is converted into an active transcriptional complex. However, the lack of suitable reporter system to follow the repression activity mediated by TCF has hampered our effort to test this hypothesis. In the current year, we have been able to establish such a system, for the first time that mimics TCF-mediated transcriptional repression. With a system that can confer both TCF-mediated transcription activation and repression available, we hope to address the fundamental question regarding how acetylation might control TCF transcriptional repression activity and how β -catenin might convert the putative transcriptional repressor complex (i.e. TCF/CBP) into an activator (i.e. TCF/CBP/ β -catenin) complex

By assessing the acetylation pattern of TCF4, we have uncovered further complexity of the TCF4 acetylation. The ability to identify additional lysine residues to be modified by acetylation might provide us with novel insight onto the function of TCF4 acetylation.

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